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NOVEL SYSTEM FOR REGULATING THE EXPRESSION OF A TRANSGENE

The present invention relates to novel compositions and methods for controlling the expression of nucleic acids in cells. The invention is more particularly suited for controlling the expression of nucleic acids in nerve cells, both in vitro and in vivo.

Controlling the expression of nucleic acids in cells is of major appeal in all spheres of biotechnology. Thus, it makes it possible, either in vitro or ex vivo, to improve the conditions under which recombinant proteins are expressed, to study the function or regulation of genes, to produce viral particles, to prepare cells which are destined for being implanted in vivo, etc. In vivo, it can make it possible to improve the properties of animal models, to study more precisely the bioavailability or toxicity of compounds or the function of genes, or else to control more effectively the production of compounds, for example for the purposes of therapy or vaccination. The compositions and methods of the invention can be used in all these spheres of biotechnology.

Various approaches for attempting to control the expression of genes have been described in the prior art. These approaches consist, in particular, in using tissue-specific transcriptional promoters or vectors. Nevertheless, the systems which have been

described do not always exhibit the expected levels of specificity, in particular in vivo, and they do not enable expression to be controlled temporally.

Gossen et al. (PNAS 89 (1992) 5547; Science 268 (1995) 1766) have described a complex system which uses the tetracycline-controlled transactivator tTa, on the one hand, and a tTa-sensitive promoter on the other hand. Although offering advantages in terms of the temporal regulation of expression, this system, as described to date, still suffers from certain drawbacks which limit its usefulness. These drawbacks are, in particular, cell toxicity, observed expression losses, the need to use two vectors, etc.

The present invention now provides a novel regulated expression system which possesses stability, specificity and manageability properties which are superior to those of the systems of the prior art.

A first aspect of the invention therefore relates to gene constructs which consist of elements whose specific nature and organization ensure tight control of gene expression.

Another aspect of the invention relates to vectors which incorporate these gene constructs, in particular viral vectors.

Another aspect of the invention therefore relates to cells which have been genetically modified by the gene constructs or vectors of the invention, to compositions which comprise them and to their use for

producing products of interest in vitro, ex vivo or in vivo.

Another aspect of the invention relates to methods and compositions for the regulated expression of a nucleic acid in cells in vitro, ex vivo or in vivo, more specifically in nerve cells. Another aspect of the invention relates to a method for the transfer and regulated expression of a nucleic acid in vivo, which method comprises the in-vivo implantation (or transplantation) of cells (in particular nerve progenitor cells) which have been genetically modified by constructs or vectors of the invention.

The present invention more specifically describes a novel regulated expression system which is based on using tTA and which exhibits improved properties. This system is particularly effective in an adenoviral context and makes possible efficient and regulated expression both in vitro and in vivo, especially in the nervous system.

The invention firstly relates, more specifically, to a nucleic acid which is characterized in that it comprises:

a) a first region which comprises a nucleic acid which encodes the tTA transactivator of the tetracycline-regulated system under the control of a moderate promoter, and

b) a second region which comprises a nucleic acid of interest under the control of a tTA-sensitive

promoter,

and in that the two regions a) and b) are arranged in the same transcriptional orientation.

More preferably, the nucleic acid according to the invention additionally comprises a third region c), which is arranged between the two regions a) and b) and which restricts transcriptional interference between regions a) and b).

Thus, the present invention results from demonstrating the advantageous properties of such constructs, in particular for controlling gene expression in vivo, in particular in nerve cells. The invention also demonstrates that the advantageous properties of these constructs result, in part, in the choice and organization of the different genetic elements among themselves.

Thus, in region a) of the nucleic acids of the invention, the promoter which is used for controlling the expression of the tTA transactivator gene is a moderate promoter. Within the meaning of the invention, the term "moderate promoter" denotes a promoter whose level of activity is regarded by the skilled person as being average, that is less than that of the strong promoters. More specifically, a moderate promoter within the meaning of the invention is a non-viral promoter which permits expression at physiological levels.

Thus, contrary to the previously described

systems (Gossen et al., PNAS 89 (1992) 5547) in which the promoter employed is a strong viral promoter (in particular hCMV), the constructs of the invention enable the tTA transactivator to be synthesized constitutively at levels which are not toxic to mammalian cells. The Applicant has now demonstrated that maintaining moderate levels of tTA in cells makes it possible to obtain a regulation of gene expression, in particular in nerve cells, which is much finer and more precise.

More preferably, the promoter employed is a cell promoter, that is a promoter which originates from a gene which is expressed in a cell. Advantageously, the promoter is a promoter which originates (or is derived) from a cell which stems from an organism of the same species as the cells in which the gene construct is intended to be introduced. Thus, when the constructs of the invention are intended for introducing nucleic acids into animals, the moderate cell promoter employed is preferably derived from a cell of an animal of the same species or of a closely related or allied species. Naturally, it is possible to use promoters which are derived from different organisms, for example a murine promoter for expression in humans, provided that the promoter employed is functional.

Still more preferably, the promoter which is used is a moderate constitutive cell promoter. Thus,

the present application demonstrates that the best properties of the system of the invention are obtained when the promoter employed is constitutive, that is ensures the presence and maintenance of stable levels of tTA in cells.

Specific examples of moderate constitutive cell promoters which are suitable for the present invention are, in particular, ubiquitous promoters such as the promoters of the housekeeping gene 3-phosphoglycerate kinase (PGK), of dihydrofolate reductase (DHFR) or else of elongation factor 1a (EF1a). Other moderate cell promoters which can be used within the context of the invention are tissue-specific promoters such as, for example, the promoters of the glial-specific GFAP ("Glial-specific fibrillary acidic protein"), neuron-specific NSE ("neuron-specific enolase"), ß-actin, ß-globin and cardiac muscle-specific MHCa ("myosis heavy chain a") genes, or else composite promoters, for example of the NRSE-PGK type.

Within the meaning of the invention, moderate promoters are therefore advantageously cell promoters which ensure a constitutive expression which is ubiquitous or localized to particular tissues or cell types and which preferably make it possible to obtain physiological levels of expression. The use of the PGK promoter (or of variants of the promoter) represents a preferred embodiment of the invention. Thus, the examples which are presented below demonstrate that

using this promoter enables expression of the nucleic acid of interest to be maintained in vivo for at least a month after introducing the construct. Thus, no decrease in the number of cells expressing the nucleic acid of interest was observed during this period. This stability of expression represents one of the significant advantages of the present invention as compared with the systems of the prior art. Thus, in transplants of progenitor nerve cells which are infected with an adenovirus which encodes ß-galactosidase under the control of a strong promoter (RSV virus LTR promoter), a clear decrease in expression levels is observed less than a month after the transplant. This therefore illustrates the advantageous properties of the invention in terms of stability, which properties are linked, unexpectedly, to using a moderate promoter for directing expression of the tTA gene.

Another important feature of the constructs according to the invention resides in the organization of the two regions a) and b). Thus, these two regions are advantageously (i) arranged in the same transcriptional orientation and (ii) separated by a region c) which restricts transcriptional interference.

The present application first of all demonstrates that, in order to improve the efficacy of the system of the invention (that is to improve the control of expression), it is particularly advantageous

to position the two cassettes a) and b) in the same transcriptional orientation. These results are all the more surprising insofar as an improved control of expression has often been observed when two cassettes are positioned in opposite orientations. By contrast, within the context of the present invention, no loss appears to occur, even in an adenoviral context, when the cassettes are in the same orientation whereas such losses had been reported in the case of a construct having an antiparallel orientation (Kojima et al., Biochem.Biophys.Res.Commun 238 (1997) 569).

On the other hand, in order to improve still further the properties of the constructs of the invention, it is particularly advantageous to introduce, between the abovementioned regions a) and b), a third region c) which restricts transcriptional interference between a) and b). The expression "transcriptional interference" refers to any influence or effect of the promoter of region a) on transcription of the nucleic acid b) and vice versa. More specifically, "transcriptional interference" refers to the difference which can exist between expression of region b) when in an isolated context, on the one hand, and when linked functionally to region a), on the other hand. Such a region c) preferably comprises a transcription terminator, preferably the UMS sequence. The UMS ("upstream mouse sequence") sequence is a genomic sequence which was identified upstream of the

c-mos murine gene (McGeady et al., DNA 5 (1986) 289). This sequence behaves like a transcription terminator. The present invention now shows that these sequences can advantageously be used for efficiently controlling gene expression in vivo, in particular in the nervous system, more specifically in an adenoviral context.

The system of the invention is therefore based on the presence, in one and the same nucleic acid, of two cassettes (regions) one of which expresses, under regulatable conditions, a transcription factor which is able to activate the expression, in the second cassette, of a nucleic acid of interest. It is therefore important, in this second cassette (region b)), to select a transcriptional promoter which is suited to the gene construct of the invention. Thus, in region b), the promoter employed is a promoter which is sensitive to the tTA transactivator, that is to say whose activity is increased in the presence of said transactivator. From a structural point of view, therefore, the promotor is a promoter which comprises, in its sequence or at a functional distance from this sequence, at least one site for binding the tTA factor (Gossen et al., 1992). This binding site or operator region (Op or tetOp) can, for example, possess the sequence detected in Figure 6 or a functional variant of this sequence. A variant can correspond, for example, to a sequence which has been genetically modified by the mutation, deletion or

addition of one or more base pairs and which retains the ability to bind the tTA transactivator. Preferably, the modifications affect less than 10% of the sequence depicted in Figure 6. In addition, the Op sequence can be present in region b) in one or more copies, for example from 1 to 10 copies, preferably from 3 to 10 copies and still more preferably from 3 to 8 copies. In one particular embodiment, the promoter employed in b) contains 7 operator sequences. Still more preferably, in order to ensure substantial control of the expression of the nucleic acid of interest, it is strongly recommended that the promoter used in b) has a very low, if not zero, basal activity. As a result, this promoter is only observed to be active in the presence of the tTA, and the regulation is therefore very tight. Advantageously, the promoter employed in b) is therefore a minimal promoter which is sensitive to the tTA transactivator. The minimal character refers to a promoter whose basal activity is very low if not zero. In general, such a promoter comprises the minimum elements which are essential for transcriptional promoter function (for example the TATA box) and lacks the other regions which are naturally involved, for example, in the strength of the promoter or in its regulation. Such promoters can be prepared from various types of promoter by means of the deletion and/or addition of elements ("silencers"). A promoter which is preferred in b) is a promoter which functions in

mammalian cells, which is essentially inactive in the absence of tTA and which is stimulated in the presence of the tTA transactivator. The term "essentially inactive" refers to the absence of any expression product (polypeptide) which can be detected by conventional techniques, in particular by enzyme assay or by the techniques of immunohistochemistry, microdialysis or HPLC. While this term does not exclude the presence of mRNA molecules which can be detected by PCR or other very sensitive detection techniques, the concentration level remains virtually insignificant.

The promoter used in b) can be of varying nature and origin and have varying properties. This is because the choice of promoter employed depends, in particular, on its sought-after use and on the gene of interest. Thus, the promoter can, for example, be derived from a promoter which is strong or weak, which is ubiquitous or specific for tissues/cells, or else specific for physiological or pathophysiological states (activity dependent on the state of cell differentiation or particular steps of the cell cycle). The promoter may be of eucaryotic, procaryotic, viral, animal, plant, artificial, human, etc., origin. Specific examples of promoters are the promoters of the TK, GH, EF1-a, APO and CMV immediate early genes, etc., or artificial promoters. For use in the present invention, these promoters are preferably rendered "minimal", that is to say dependent on the presence of

the tTA. For this, the original promoters can be digested with enzymes and tested for their activity, preferably after they have been functionally coupled to one or more Op sequences. The number of tetOp sequences, as well as their distance in relation to the chosen promoter, can be adapted by the skilled person so as to ensure that expression of said promoter is strictly tTA-dependent. Preferably, the promoter used in b) is a minimal promoter from the cytomegalovirus (CMV) immediate early gene, preferably from human CMV (hCMV), as described in Corti et al (Neuroreport 7 (1996) 1655), which publication is hereby incorporated by reference.

The present invention can be used for expressing a variety of nucleic acids of interest depending on the sought-after applications. Thus, in region b), the nucleic acid of interest is advantageously a nucleic acid which encodes a protein or a polypeptide of interest. Within the meaning of the present invention, products of interest which may be mentioned more specifically are enzymes, blood derivatives, hormones such as growth hormones, cytokines, lymphokines: interleukins, interferons, TNF, etc. (French patent No. 92 03120), growth factors, for example angiogenic factors such as VEGF or FGF, neurotransmitters or their precursors or enzymes for synthesizing them (tyrosine hydroxylase: TH), trophic factors, in particular neurotrophic factors for

treating neurodegenerative diseases, traumas which have damaged the nervous system or retinal degenerations: BDNF, CNTF, NGF, IGF, GMF, aFGF, NT3, NT5 or HARP/pleiotrophin, or bone growth factors, hematopoietic factors, etc., dystrophin or a minidystrophin (French patent No. 91 11947), genes encoding factors involved in coagulation: factors VII, VIII and IX, suicide genes (thymidine kinase and cytosine deaminase), proteins involved in the cell cycle, such as p21, or other proteins which inhibit dependent kinases, Rb, Gas-1, Gas-6, Gas-3, Gad 45, Gad 153, cyclins A, B and D, or else the GAX protein which restricts proliferation of cells in smooth muscles (treatment of restenosis), apoptosis-inducing proteins or other tumor suppressors such as p53, Bax, BclX-s, Bad or any other antagonist of Bcl2 and BclX-1, genes for hemoglobin or other protein transporters, genes corresponding to the proteins involved in the metabolism of lipids, of the apolipoprotein type selected from the apolipoproteins A-I, A-II, A-IV, B, C-I, C-II, C-III, D, E, F, G, H, J and apo(a), enzymes of metabolism such as lipoprotein lipase, hepatic lipase, lecithin cholesterol acyltransferase, 7-alpha-cholesterol hydroxylase or phosphatidyl acid phosphatase, or else lipid-transferring proteins such as cholesterol ester-transferring protein and phospholipid-transferring protein, an HDL-binding protein, or else a receptor which is selected from the

LDL receptors, the chylomicron remnant receptors and the scavenger receptors, etc.

Among the products of interest, it is important to emphasize antibodies, single-chain variable antibody fragments (ScFv) or any other antibody fragment which possesses recognition capabilities for its use in immunotherapy, for example for treating infectious diseases, tumors (anti-RAS, anti-p53 or anti-GAP antibodies), or autoimmune diseases such as multiple sclerosis (antiidiotype antibodies).

In a non-limiting manner, other proteins of interest are soluble receptors such as the soluble CD4 receptor or the soluble TNF receptor in the case of anti-HIV therapy, or the soluble acetylcholine receptor in the case of myasthenia treatment; peptides which are substrates or inhibitors of enzymes, or else peptides which are agonists or antagonists of receptors or of adhesion proteins as, for example, in the case of the treatment of asthma, of thrombosis and of restenosis: artificial, chimeric or truncated proteins. Hormones of principle interest which may be mentioned are insulin, in the case of diabetes, growth hormone and calcitonin.

The nucleic acid can also be a gene or an antisense sequence whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can, for example, be transcribed, in the target cell,

into RNA which is complementary to the cellular mRNAs and thereby block the translation of the latter into protein, in accordance with the technique described in European patent No. 140 308. The therapeutic genes also comprise the sequences which encode ribozymes which are able to selectively destroy target RNAs (European patent No. 321 201).

The nucleic acid can also contain one or more genes encoding an antigenic peptide which is able to generate an immune response in humans or animals. In this particular embodiment, the invention therefore makes it possible either to develop vaccines or immunotherapeutic treatments which are applied to humans or animals, in particular against microorganisms, viruses or cancers. The peptides can, in particular, be antigenic peptides which are specific for the Epstein Barr virus, for the HIV virus, for the hepatitis B virus (European patent No. 185 573), for the pseudo-rabies virus, for the "syncitia-forming virus" or for other viruses, or else antigens which are tumor-specific such as the MAGE proteins (European patent No. 259 212).

The nucleic acid can also encode a product which is toxic for cells, in particular which exhibits conditional toxicity (i.e. thymidine kinase, cytosine deaminase, etc.).

Other genes which are of interest have, in particular, been described by McKusick and V.A.

Mendelian (Inheritance in man, catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes, Eighth edition. John Hopkins University Press (1988)) and in Standbury, J.B. et al. (The metabolic basis of inherited disease, Fifth edition. McGraw-Hill (1983)). The genes of interest cover the proteins which are involved in the metabolism of amino acids, of lipids and of other constituents of the cell.

On the other hand, region b) can also comprise a transcription terminator which is located 3' of the nucleic acid of interest. Finally, the nucleic acid can contain several coding regions which are, where appropriate, separated by an IRES, making it possible to produce several products of interest.

Since the constructs according to the invention are particularly well suited for regulating expression in the nervous system, the nucleic acids of interest more especially encode neurotransmitters or their precursors or enzymes for synthesizing them (tyrosine hydroxylase: TH), or trophic factors, in particular neurotrophic factors for treating neurodegenerative diseases, traumas which have damaged the nervous system or retinal degenerations.

In one particular embodiment, the invention relates to a nucleic acid which is characterized in that it comprises:

a) a first region which comprises a nucleic acid which encodes the transactivator of the

tetracycline-regulated system (tTA) under the control of the promoter of the PGK gene, and

b) a second region which comprises a nucleic acid of interest which encodes, in particular, human tyrosine hydroxylase under the control of the minimal CMV promoter which has been modified so as to include from 1 to 10, preferably from 3 to 8, in particular 7, tetOp sequences,

c) a third region which comprises a UMS sequence,

and in that the two regions a) and b) are arranged in the same transcriptional orientation.

The nucleic acid of the invention can be either DNA or RNA. It can, more particularly, be a genomic DNA (gDNA) or a complementary DNA (cDNA). The nucleic acids of the invention can be prepared using techniques which are well known to the skilled person and which involve, for example, the synthesis of nucleic acids, the screening of libraries, ligation, cutting with enzymes, amplification, cloning, etc. as illustrated in the examples (see Maniatis et al.). Thus, the different regions a), b) and c) can be prepared separately and then assembled functionally, that is to say in the same transcriptional orientation. The space between regions a) and b) may vary without this harming the efficiency of the constructs of the invention. Thus, these regions can be separated by from 0 to 3000 bp, for example, more generally by from 0 to

1000 bp, advantageously by less than 500 bp. This distance between regions a) and b) is generally determined by the length of region c) (when it is present) and by the cloning capacity of the vector employed. It can easily be adjusted by the skilled person. The nucleic acids of the invention can comprise elements of varied origin, in particular originating from procaryotic, eucaryotic (animal, plant, viral, etc.), synthetic or semisynthetic nucleic acids.

The present invention also relates to any vector which comprises a nucleic acid as defined above. The vector can be any vector known to the skilled person, such as a plasmid, a cosmid, a phage, a YAC, a HAC, a transposon, an episome, a virus, etc. A type of vector which is preferred according to the invention is represented by viral vectors such as adenoviruses, AAVs, herpesvirus, vaccinia virus or some retroviruses (in particular lentiviruses). The virus is still more preferably a virus which possesses a tropism for nerve cells, in particular nerve cell progenitors. In this regard, a vector which is particularly preferred within the context of the present invention is an adenovirus.

The adenoviruses which are used for implementing the present invention are advantageously recombinant defective adenoviruses, that is whose genome includes a heterologous nucleic acid and which are unable to replicate autonomously in cells. More preferably, the adenoviruses of the invention are at

least defective for all or part of the E1 and E3 regions.

The recombinant defective adenoviruses can also be 3rd generation recombinant defective adenoviruses, that is recombinant adenoviruses which are defective for all or part of the E1 and E4 regions and possibly for the E3 region.

Particular variants of the invention consist of using adenoviruses which carry deletions which affect all or a functional part of the following regions:

-E1, E4 and E3,

-E1, E4 and E2,

-E1, E4, E2 and E3,

- the above regions as well as all or part of the genes which encode the late functions of the adenovirus (L1 to L5), or else

- all the coding regions of the virus.

The genomic structure of the adenoviruses has been amply described in the literature. In this regard, the genome of the adenovirus Ad5 has been completed in sequence and is accessible in databases (see, in particular, GeneBank M73260). Similarly, parts, if not the entirety, of other adenoviral genomes (Ad2, Ad7, Ad12, canine adenovirus CAV-2, etc.) have also been sequenced. Furthermore, the construction of recombinant defective adenoviruses has also been described in the literature. Thus, applications WO 94/28152, WO 95/02697

and WO 96/22378, for example, describe various deletions in the E1 and E4 regions. Similarly, Application WO 96/10088 describes vectors which carry a modification at the level of the lva2 gene, while Application WO 94/26914 describes adenoviruses of animal origin and WO 95/29993 describes deletions which affect the E2 region of the adenovirus.

Advantageously, the recombinant adenovirus which is used within the context of the invention comprises a deletion in the E1 region of its genome, with this deletion affecting the E1a and E1b regions. Deletions affecting nucleotides 454-3328, 382-3446 or 357-4020 (by reference to the Ad5 genome) may be mentioned as specific examples.

Furthermore, the deletion in the E4 region preferably affects all the open reading frames, for example deletions 33466-35535 or 33093-35535, or only a part of the E4 region (ORF6 or ORF3, for example), as described in Applications WO 95/02697 and WO 96/22378, which are hereby incorporated by reference.

The construction of the adenoviruses which additionally lack late functions ("minimum" vector) or all the coding regions ("gutless" vector) has been described, for example, by Parks et al. PNAS 93 (1996) p. 13565 and Lieber et al., J. Virol. 70 (1996) p. 8944.

The nucleic acids of the invention can be inserted into various sites on the recombinant

adenoviral genome. They can be inserted within the E1, E3 or E4 region, either by replacing deleted sequences or over and above the normal sequences. They can also be inserted in any other site apart from the sequences which are required in cis for producing the viruses (ITR sequences and encapsidation sequence).

Furthermore, the recombinant adenoviruses can be of human or animal origin. The adenoviruses of human origin which may preferably be mentioned are those which are classed in the C group, in particular type 2 (Ad2) and type 5 (Ad5) adenoviruses; or type 7 (Ad7) or type 12 (Ad12) adenoviruses. Of the various adenoviruses of animal origin, those which may preferably be mentioned are the adenoviruses of canine origin, in particular all the strains of the CAV2 adenoviruses [Manhattan strain or A26/61 (ATCC VR-800), for example]. Other adenoviruses of animal origin are mentioned, in particular, in Application WO 94/26914, which is hereby incorporated by reference.

The recombinant adenoviruses are produced in an encapsidation cell line, that is a line of cells which are able to complement in trans one or more of the functions which are deficient in the recombinant adenoviral genome. An example of an encapsidation cell line which is known to the skilled person and which may be mentioned is the cell line 293 into which a part of the adenovirus genome has been integrated. More precisely, cell line 293 is a line of human kidney

embryonic cells which contain the left-hand end (approximately 11-12%) of the adenovirus serotype 5 genome (Ad5) comprising the left-hand ITR, the encapsidation region, the E1 region, including E1a and E1b, the region encoding the pIX protein and a part of the region encoding the pIVa2 protein. This cell line is able to transcomplement recombinant adenoviruses which are defective for the E1 region, that is which lack all or part of the E1 region, and to produce viral stocks having high titers. At a permissive temperature (32°C), this cell line is also able to produce stocks of virus which additionally contain the temperature-sensitive E2 mutation. Other cell lines which are able to complement the E1 region have been described, with these cell lines in particular being based on A549 human lung carcinoma cells (WO 94/28152) or on human retinoblasts (Hum. Gen. Ther. (1996) 215). In addition, cell lines which are able to transcomplement several adenovirus functions have also been described. Particular mention may be made of cell lines which complement the E1 and E4 regions (Yeh et al., J. Virol, Vol. 70 (1996) pp. 559-565; Cancer Gen. Ther. 2 (1995) 322; Krougliak et al., Hum. Gen. Ther. 6 (1995) 1575) and of cell lines which complement the E1 and E2 regions (WO 94/28152, WO 95/02697, WO 95/27071) or of cell lines which are derived from these cell lines and which can be used for producing minimal adenoviruses, in particular because they additionally express a site-

specific recombinase activity which is involved in constructing such viruses.

Recombinant adenoviruses are generally produced by introducing the viral DNA into the encapsidation cell line, with the cells then being lysed after about 2 or 3 days (the kinetics of the adenoviral cycle being from 24 to 36 hours). For implementing the method, the viral DNA which is introduced can be the complete recombinant viral genome, which can, where appropriate, have been constructed in a bacterium (WO 96/25506) or in a yeast (WO 95/03400), and which is then transfected into the cells. The viral DNA can also be in the form of a recombinant virus which is used when infecting the encapsidation cell line. The viral DNA can also be introduced in the form of fragments, with each of the fragments carrying a part of the recombinant viral genome and a region of homology which makes it possible, after the fragments have been introduced into the encapsidation cell, to reconstitute the recombinant viral genome by means of homologous recombination between the different fragments.

After the cells have been lysed, the recombinant viral particles can be isolated by any known technique such as centrifugation in a cesium chloride gradient or chromatography. An alternative method has, in particular, been described in the Application FR 9608164, which is hereby incorporated by

reference.

The invention additionally relates to a composition which comprises a vector of the above-defined type, preferably a recombinant defective adenovirus of the above-defined type. In this embodiment, the compositions according to the invention can comprise varying quantities of recombinant adenoviruses, which quantities can readily be adjusted by the skilled person in accordance with the envisaged applications (in vitro, ex vivo or in vivo, for example). In general, the compositions comprise in the order of from 10^5 to 10^{15} v.p. of recombinant adenovirus, preferably from 10^7 to 10^{12} v.p. The term v.p. corresponds to the number of viral particles which are present in the compositions.

The vectors and compositions of the invention can either be used directly, in vitro or in vivo, for the transfer and regulated expression of nucleic acids in the cells or else used for introducing nucleic acids into the cells with a view to these cells being implanted (or transplanted) in vivo.

The invention also relates to any cell which comprises a nucleic acid or a vector as defined above. Preferably, the cell is a mammalian cell, preferably a human cell. In one particularly preferred embodiment, the cell is a nerve cell, in particular a progenitor nerve cell. An example of such a population of cells is the human nerve progenitor cell as described by Buc et

al. (Neurobiol.Dis. 2 (1995) 37).

In this regard, one particular embodiment of the invention comprises a nerve cell which is genetically modified by a recombinant adenovirus which comprises a nucleic acid as defined above.

The invention also relates to any composition which comprises cells such as those described above.

The invention additionally relates to the use of a nucleic acid or a vector or a composition, as described above, for preparing a composition which is intended to express a nucleic acid of interest in vivo.

The invention also relates to the use of a nucleic acid or a vector or a composition, as described above, for preparing a composition which is intended to express a nucleic acid of interest in the nervous system in vivo.

In one particular embodiment, the invention relates to a method for the regulated expression of nucleic acids in the nervous system, which method comprises implanting a cell, such as defined above, into the nervous system of a patient and controlling expression by administering tetracycline or a tetracycline analog to the patient.

The compositions of the invention can be presented in a variety of forms, such as solutions, gels, powder, etc. They are generally in the form of solutions which are preferably sterile, for example isotonic saline (monosodium or disodium phosphate,

sodium, potassium, calcium or magnesium chloride, etc., or mixtures of these salts) solutions or of dried, in particular lyophilized, compositions which enable solutions to be constituted by means of adding sterilized water or physiological saline, as the case may be. Use can be made of other excipients such as a hydrogel. This hydrogel can be prepared from any biocompatible and non-cytotoxic polymer (homopolymer or heteropolymer). Such polymers have, for example, been described in Application WO 93/08845. In addition, the compositions according to the invention can be presented in any type of suitable container, such as a bottle, a tube, an ampoule, a pocket, a syringe, a small flask, etc.

As indicated above, the compositions of the invention can be used in vitro, ex vivo or in vivo.

For an in vitro or ex vivo use, the cells can simply be incubated in any suitable container (plate, dish, pocket, etc.) in the presence of a vector composition as described above. For this type of application, the compositions comprising adenoviruses can be used at multiplicities of infection (MOIs) of, for example, between 10 and 5000 v.p., preferably between 100 and 2000 v.p., per cell.

For an in vivo use, the compositions of the invention can be formulated with a view to being administered by the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous,

intraocular, transdermal or intratumoral route, etc. In this application, in particular for cellular compositions, the compositions employed advantageously comprise from 10^4 to 10^6 cells, more preferably from 10^5 to 10^6 cells. Furthermore, the implanted compositions preferably comprise a high density of cells (from about 10^5 to 10^6 per µl). Thus, the results which are presented in the examples show that using a high density of cells enables the implant to take more readily and promotes the survival of the implanted cells. Furthermore, in order to promote the survival of the in vivo implants still further, it is also possible to administer, at the same time or previously, if not subsequently, one or more immunosuppressive compounds or an immunosuppressive treatment (Neuroscience 78 (1997) 685).

In this regard, the invention also relates to a method for transferring a nucleic acid in vivo, which method comprises administering a composition such as described above. For example, the invention can be used in animals for creating pathological models or studying the regulation of genes or else in humans, in labeling or bioavailability studies or for medical purposes.

For regulating expression, the compositions of the invention advantageously comprise tetracycline or any tetracycline analog which is able to affect the activity of the tTA transactivator. An example of such an analog is doxycycline (Kistner et al., PNAS 93

(1996) 10933). Other analogs which can be used in the present invention are described, for example, by Alvarez et al. (Gene Ther. 4 (1997) 993), which publication is hereby incorporated by reference. The analog doses can readily be adjusted by the skilled person in dependance on the analog and the constructs employed. As an indication, doses of 0.1 ng/ml of doxycycline are sufficient to completely block, in vitro, nucleic acid expression in the constructs of the invention.

The present invention is particularly suitable for treating (i.e. for partially or completely suppressing) neurodegenerative diseases such as, in particular, Parkinson's disease (PD). PD is a disease which is characterized by the progressive loss of dopaminergic neurons in the substantia nigra. While the disease is treated with L-DOPA, in an attempt to control the symptoms of the disease, the efficacy of this treatment declines as the disease progresses. The present application offers an advantageous alternative for treating this disease by introducing a gene (tyrosine hydroxylase, TH) which is involved in synthesizing dopamine in vivo. Experiments which have been carried out by means of long-term pharmacological treatment with dopaminergic compounds have demonstrated that these treatments induce an unregulated, generalized effect which leads, in most cases, to very undesirable fluctuations in motor neuron response. The

present invention is particularly suitable for this type of situation since it permits a control of L-DOPA synthesis which is both fine and local, which types of control are essential for obtaining a motor response which physiologically reproduces the complex mechanisms of dopaminergic stimulation.

The present application will be described in more detail with the aid of the examples which follow and which should be regarded as being illustrative and not limiting.

FIGURE LEGENDS

Figure 1: Diagram of the structure of the genome of an adenovirus containing a nucleic acid according to the invention. The inverted terminal repeat (ITR) sequences and the encapsidation sequence (Ψ) are shown. pIX represents the sequence encoded by the adenoviral IX protein.

Figure 2: Panel (a): Infection of a human nerve progenitor with the adenovirus AdPGK.tet.hTH-1 at various MOIs. The values shown are means of four experiments.

Panels (b) and (c): Production of TH in vitro in human nerve progenitors, as measured by immunocytochemistry seven days after an MOI = 140 infection. Panel (b) without treatment, and panel (c)

after treatment in the presence of doxycycline (10 ng/ml) immediately after infection and up to day 7. The magnifications are 360 (panel (b)) and 220 (panel (c)).

Figure 3: Kinetics of the disappearance (a) and resumption (b) of hTH activity in cultures which are infected with adenovirus AdPGK.tet.hTH1 (MOI = 40) following the addition (a) or withdrawal (b) of doxycycline (5 ng/ml) on day 0 (5.5 days after infection). The hTH activity in untreated cultures was determined to be 100% on day 0. (c) Effect of different doses of doxycycline on the expression of hTH, as determined on day 11 after infection. The values are the means of at least 4 experiments.

Figure 4: Immunoreactivity to TH in the intrastriatal implants of human nerve progenitors infected with adenovirus AdPGK.tet.hTH-1 (MOI = 40), as measured by anti-TH immunohistochemistry on 15 µm freeze-dried brain sections [lacuna] from (a) untreated animals and (b) an animal which was treated daily with doxycycline. The violet staining (a) (b) reveals the presence of human cells which hybridize with the digoxygenin-labeled alu probe, subsequent to incubation with an alkaline phosphatase-coupled anti-digoxygenin antibody and then staining with a specific substrate. Magnification 110.

Figure 5: TH immunoreactivity in the implants of human nerve progenitors infected with adenovirus AdPGK.tet.hTH1 (MOI = 40) as measured by anti-TH immunohistochemistry, and in-situ hybridization using a 35 sulfur-labeled HTH-1-specific antisense oligonucleotide, as carried out on 15 µm freeze-dried brain sections from (a) an untreated animal and (b) an animal treated daily with doxycycline. These sections were counterstained with cresyl violet. Magnification 450.

Figure 6: Nucleotide sequence of the tTA gene (A); of the UMS sequence (B); of the Op sequence (C) and of the minimal CMV promoter (D).

EXAMPLES

I - Materials and methods

1. Construction of the adenovirus AdPGK.Tet. HTH-1.

A DNA fragment comprising the sequence encoding the transactivator of the tetracycline-regulated system (tTA) and the human cytomegalovirus minimum promoter (hCMV) was excised from the plasmid pUMS-luc (Corti et al. Neuroreport 7 (1996) 1655) by digesting with the enzymes XhoI and ScaI and inserted into the shuttle plasmid pCMVlucIX (supplied by M.C. Geoffroy), which was digested with enzymes BglII/ClaI.

This resulted in the vector ptetIX. A BglII/HindIII fragment containing the cDNA for human tyrosine hydroxylase I (hTH-1) (Grima B. et al. Nature 1987, 326:707-711) followed by the SV40 virus polyadenylation signal was inserted between the ClaI and EcoRV sites of vector ptetIX, thereby generating vector ptetIXhTH-1. The human cytomegalovirus immediate early promoter (hCMVp), controlling the expression of tTA in vector ptetIXhTH, was replaced with the ubiquitous murine promoter of the gene for phosphoglycerate kinase (PGK) (Adra et al., Gene 1987; 60-65). A XhoI/SplI fragment containing the PGK promoter was ligated between the SpeI and SplI sites of vector ptetIXhTH-1 in order to generate the plasmid pPGKtetIXhTH-1. For homologous recombinations (Stratford et al. J. Clin. Invest. (1992) 626), the vector pPGKtetIXhTH-1 was linearized with XmnI and cotransfected together with the large ClaI fragment of the adenovirus Adßgal into the 293 cell line, which transcomplements the E1 region. The recombinant adenovirus AdPGK.tet.hTH-1 was purified twice by plaque assay. A viral stock was obtained by amplifying on the 293 cells and then purifying on a gradient and cesium chloride followed by a Sephadex column. The viral titer, determined by plaque titration on 293 cells, is 3.5×10^{10} pfu/ml.

2. Generation and culture of human nerve progenitor cells.

Primary cultures of human embryonic brains were obtained and propagated in vitro in a medium without serum which was supplemented with basal FGF, as previously described (Buc et al., Neurobiol. 10 (1995) 37).

3. Infection of human nerve progenitor cells.

For the in vitro studies, the cells were seeded onto 12-well tissue culture plates at a density of from 6 to 7 × 10^5 cells per well and incubated with the virus at the chosen multiplicity of infection (MOI) in 400 µl of a defined medium lacking serum (DS-FM). After 6 hours, the supernatants were removed and replaced with an equivalent volume of DS-FM. At various times after infection, the cells are harvested and centrifuged (4000 RPM, 5 min), and the pellets are frozen at -80°C for the enzyme assays (Reinhardt et al., Life Sciences (1986) 21-85).

For the transplantations, the cells were seeded on 6 cm diameter tissue culture plates at a density of 5 × 10^6 cells per plate and incubated for 6 hours with the AdPGK.tet.hTH-1 virus at an MOI of 40 pfu per cell. On the day following infection, the cells were harvested as previously described (Sabaté et al., Nature Genetics, 9 (1995) 256), resuspended in DS-FM medium at a density of 4 × 10^5 cells per µl and

stored on ice during all the implantation operations.

4. Lesions and intracerebral implantations.

From five to six weeks before the intracerebral implantations, 6-hydroxydopamine (6-OHDA) was injected stereotactically into the left ascending mesostriatal dopaminergic tract of adult Sprague-Dawley (Charles-River) rats, as previously described (Horellou et al., Neuron 5 (1990), 393). Sixteen rats were implanted under anesthesia produced by 800 µl per kg of a mixture of ketamine (UVA) and Rompun (Bayer) in a ratio of 1:1. One µl of cellular suspension was injected, using a 10 µl Hamilton syringe, into each of two sites on the injured striatum at the following stereotactic coordinates: + 0.7 anterior from the bregma (AB), + 2.5 lateral from the median line (ML), 5 ventral on the dural surface (V), and + 1.5 AB, - 2.5 ML, 5 V (starting line set at 0). The animals were injected daily with 10 mg of cyclosporin per kg.

5. In situ hybridization and immuno-histochemistry

Four weeks after the implantation, the animals were perfused as previously described (Sabaté et al. loc. cit.). The brains were recovered, fixed in 4% pfa, and stored in PBS medium containing 20% sucrose. 15 µm sections were obtained subsequently.

The human cells were detected by

hybridization using a human-specific alu primer which was labeled with digoxigenin-tagged nucleotides (5'-XTTgCAgTgAgCCgAgATCgCgCC-3'). After an initial denaturation step (20 minutes in 95% formamide, 0.1 × SSC at 75° and with shaking), sections were treated as previously described (Dumas et al., J. Chem. Neuroanat. 5 (1992) 11). Human TH-1 RNA was detected in the implants by in situ hybridization using a 35 sulfur-labeled oligonucleotide which was directed against exon 1 of human TH (5'-TgCCTgCTTggCgTCCAgCTCAgACA-3'). The hybridization conditions are identical to those described by Lanièce et al. (J. Neurochem. 66 (1996) 1819). For the immunohistochemistry, the sections were treated in accordance with standard techniques.

II - Results

1. Generating an adenovirus which encodes human TH-1 under the control of a regulatory system according to the invention (AdPGK.tet.hTH-1).

In order to obtain regulated expression of the hTH-1 transgene using a single adenoviral vector, the tet-off negative regulatory system using tetracycline, which was initially based on using two vectors, was modified. The tetracycline-sensitive tTA transactivator and human TH-1 cDNA were inserted into an Ad5 adenovirus skeleton from which the E1 and E3 regions had been deleted. In the vector AdPGK.tet.hTH-

1, tTA expression is under the control of the ubiquitous promoter of the murine phosphoglycerate kinase (PGK) gene, whereas transcription of the human TH is controlled by a tTA-sensitive minimum promoter (the CMV promoter). The UMS sequence was inserted between the tTA and the CMV promoter in order to restrict transcriptional interference between the two transcription units.

2. Infection of human nerve progenitors with AdPGK.tet.hTH-1: regulated expression in vitro.

In order to determine whether the AdPGK.tet.hTH-1 adenovirus was able to give rise to active synthesis of TH in human nerve progenitors, cells were infected at various multiplicities of infection (Figure 2a). The results obtained show that TH activity increases in the infected cells as a function of the vector dose employed, thereby demonstrating that the AdPGK.tet.hTH-1 adenovirus is functional.

The possibility of controlling the expression of the hTH-1 gene with doxycycline was studied in the adenovirus-infected cells. When the antibiotic was added (10 ng/ml) to the culture medium immediately after infection, it was not then possible to demonstrate any TH activity at any time, even when the highest virus dose was used. Furthermore, no cell exhibiting TH immunoreactivity was identified (Figures

2b and 2c).

The possibility that expression of the gene could also be arrested subsequently in the cells which were already expressing the transgene was then tested. For this, doxycycline was added to the culture medium 5.5 days after infection, when TH activity was already detectable in the cells. The results obtained show that the enzymatic activity declines progressively and disappears almost completely 5.5 days after adding the doxycycline (Figure 3a). Thus, transcription of the TH-1 gene, under the conditions of the invention, in human nerve progenitors, can be regulated negatively in a very efficient manner.

In order to test whether it is possible to reinduce transgene activity, TH activity was monitored after doxycycline had been withdrawn from the culture medium of the infected cells (Figure 3d). The results obtained show that TH activity increases progressively in the cells, thereby indicating that inhibition of the transgene with doxycycline is reversible.

In order to determine the minimum dose of the antibiotic required for inhibiting expression of the gene, the cells were then incubated with varying concentrations of doxycycline immediately following infection (Figure 3c). A concentration of 0.1 ng/ml is sufficient to prevent the appearance of TH activity whereas TH activity can be detected at a dose of 0.01 ng/ml.

3. Transplantation into the brain of human nerve progenitors which are infected with the AdPGKtet.hTH-1 adenovirus: control of expression in vivo.

This example demonstrates the ability of the system of the invention and, in particular, of the adenovirus-infected human nerve progenitor cells to mediate expression of human TH in vivo, and also the ability of the system of the invention to regulate this in vivo expression, in particular using doxycycline. The cells were infected with the AdPGK.tet.hTH-1 adenovirus and then implanted into the striatum of rats which had undergone a unilateral lesion of the dopaminergic pathway which had been caused by injecting the toxin 6-OHDA. In order to facilitate the implant taking, a large number of cells (8×10^5), suspended at high density ($4 \times 10^5/\mu l$), was implanted into each animal. After the implantation had taken place, the animals were either not treated (n = 8) or treated (n = 8) daily with doxycycline (1 mg/ml in water). Four weeks after the implantation, the rats were sacrificed and the brains were analyzed for TH expression.

All the brains tested contain viable implants of human cells as demonstrated by in situ hybridization with an oligonucleotide probe which is directed against the human-specific alu repeat sequence (Figure 4a). No differences in the size or appearance of the implants were observed between the animals which were or were not treated with doxycycline.

The implants in the untreated animals exhibit substantial TH immunoreactivity (Figure 4a). The number of cells expressing TH in the implants was thus estimated to be between 5 and 10%. A similar percentage of cells which had been recultured, after the implantation, for six days in a medium without serum also exhibits TH immunoreactivity. These results clearly demonstrate expression of the gene in vivo under the same conditions as in vitro.

On the other hand, no cell exhibiting TH immunoreactivity was detected in any of the implanted animals which had been given doxycycline (Figure 4b). This indicates that doxycycline efficiently inhibits the appearance, in the implants, of a sufficient quantity of TH to be detected by immunohistochemistry.

In order to establish whether the effect of doxycycline reflects tight control of the gene expression at the transcriptional level, an in situ hybridization was carried out on sections using an oligonucleotide which was specific for TH-1 messenger RNA. The implants in the untreated animals were labeled to a very substantial extent. The labeling was co-located with the immunoreactive cells (Figure 5a). On the other hand, no specific labeling was detected in the implants of the treated animals (Figure 5b).

In conclusion, it can be said that doxycycline efficiently inhibits transcription of the transgene in the system of the invention in an

adenoviral context which is introduced by implanted human nerve progenitors.